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Rat Bladder Cell-Mediated Mutagenesis of Chinese Hamster V79 Cells and Metabolism of Benzo(a)Pyrene

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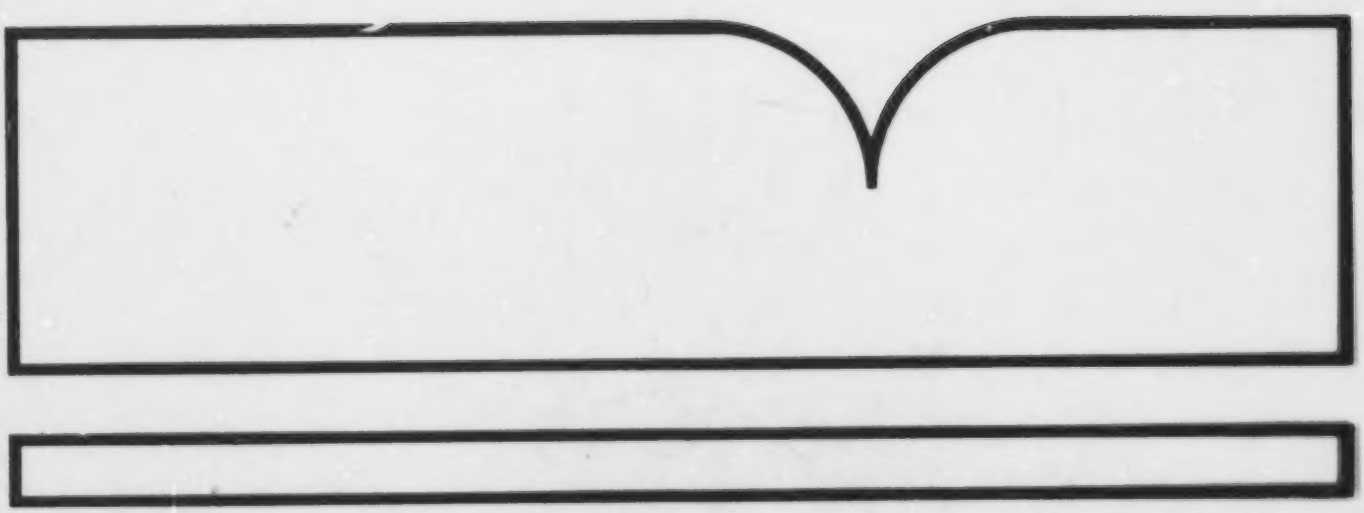

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16. ABSTRACT

Primary rat bladder epithelial cells were coculivated with Chinese hamster V79 cells in the presence of carcinogens, and the induction of 6-thioguanine resistance in the V79 cells was used as a marker of cell-mediated mutagenesis. The carcinogens dimethylnitrosamine, 7,12-dimethylbenz[a]anthracene, and benzo[a]pyrene (BP) were mutagenic to V79 cells in the presence of bladder cells but not in their absence. Analysis of BP metabolites formed by bladder cells indicated that 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene, 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene, benzo[a]pyrene-3,6-quinone, and 9-hydroxybenzo[a]pyrene were the major organic-soluble metabolites formed. Glucuronide and sulfate conjugates of BP metabolites were also produced by bladder cells. Mutagenesis data from the rat bladder system and previous data from rat liver and lung cell-mediated mutagenesis systems indicate that the cell-mediated mutagenesis approach may provide a useful approach for studying the organotropic effect of chemical carcinogens. Furthermore the finding that rat bladder epithelium can metabolize some carcinogens offers new possibilities for the mechanism of initiation of bladder cancer.

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Rat Bladder Cell-Mediated Mutagenesis of Chinese Hamster V79 Cells and Metabolism of Benzo[a]pyrene [1, 2]

Robert Langenbach, [3, 4, 5] **Linda Malick,** [5] *and* **Stephen Nesnow** [3, 6]

ABSTRACT—Primary rat bladder epithelial cells were cocultivated with Chinese hamster V79 cells in the presence of carcinogens, and the induction of 6-thioguanine resistance in the V79 cells was used as a marker of cell-mediated mutagenesis. The carcinogens dimethylnitrosamine, 7,12-dimethylbenz[a]anthracene, and benzo[a]pyrene (BP) were mutagenic to V79 cells in the presence of bladder cells but not in their absence. Analysis of BP metabolites formed by bladder cells indicated that 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene, 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene, benzo[a]pyrene-3,6-quinone, and 9-hydroxybenzo[a]pyrene were the major organic-soluble metabolites formed. Glucuronide and sulfate conjugates of BP metabolites were also produced by bladder cells. Mutagenesis data from the rat bladder system and previous data from rat liver and lung cell-mediated mutagenesis systems indicate that the cell-mediated mutagenesis approach may provide a useful approach for studying the organotropic effect of chemical carcinogens. Furthermore, the finding that rat bladder epithelium can metabolize some carcinogens offers new possibilities for the mechanism of initiation of bladder cancer.—JNCI 1981; 66:913-917.

We are interested in developing cell culture systems in which the mechanism of carcinogenesis and the organ specificity of chemical carcinogens can be studied (*1-4*). Many carcinogens exist in the environment in a nonreactive form and must be metabolically activated to manifest their biologic activity (*5, 6*). Because cell homogenates produce metabolite and DNA adduct profiles that differ from those produced in vivo or by intact cells (*7-10*), we utilize intact cells from the respective tissue to study organ specificity. In these systems, primary cultures of cells from the tissues or organs are cocultivated with Chinese hamster V79 cells, and mutation of the V79 cells is used as a measure of the ability of the tissues to activate the chemical to carcinogenic or mutagenic intermediates. The development of V79 mutagenesis systems mediated by rat liver cells (*1*) and rat lung cells (*2*) has been reported. Cell type specificity (*3*) and organ specificity (*4*) in the metabolism of chemical carcinogens to mutagenic intermediates has been shown. In addition, the liver cell-mediated system has proved useful as a sensitive short-term test for detecting certain classes of chemicals (*11, 12*).

In the present study, we extend this methodology to a rat bladder cell-mediated mutagenesis system. The mutagenic activities of three potent carcinogens, DMN, DMBA, and BP, in the rat bladder cell system are presented. Furthermore, the metabolism of BP is analyzed by the use of high-pressure liquid chromatography. The implications of the metabolic activity of bladder epithelium in relation to various theories for bladder cancer initiation are discussed.

MATERIALS AND METHODS

Bladder cell preparation.—Noninbred male 6- to 8-week-old Sprague-Dawley rats were anesthetized with Nembutal, and the bladders were removed and placed in sterile PBS. Approximately 20 animals were used per experiment. The bladders were minced into approximately 0.25-cm^2 pieces and placed in a 100-ml flask with 50 ml of a 0.5 m*M* EGTA solution containing 0.25% trypsin and 30 U/ml collagenase in PBS. The bladder tissue was stirred slowly for 5 hours at 4°C. Rapid stirring resulted in aggregation of the tissue. The tissue was removed and stirred at 37°C for 15 minutes. Stirring for longer times at 37°C increased the number of fibroblast-like cells and red blood cells. Fibroblast and red blood cell contamination was to be avoided because fibroblasts could contribute to metabolism of DMBA and BP but not to DMN metabolism (*3*), while large numbers of red blood cells were toxic to V79 cells. After the incubation at 37°C, the flask was shaken gently, and the supernatant containing the cells was removed and mixed with an equal volume of complete medium before centrifugation at 50×*g* and 4°C for 10 minutes. The bladder cells were resuspended in complete medium and counted on a hemacytometer. About 10^6 cells were obtained per rat bladder, and the viability was 90-95% by trypan blue dye exclusion. The medium used in all studies was Williams Medium E

ABBREVIATIONS USED: BP = benzo[a]pyrene; BP-4,5-diol = 4,5-dihydro-4,5-dihydroxybenzo[a]pyrene; BP-7,8-diol = 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene; BP-9,10-diol = 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene; BP-3-phenol = 3-hydroxybenzo[a]pyrene; BP-9-phenol = 9-hydroxybenzo[a]pyrene; BP-1,6-quinone = benzo[a]pyrene-1,6-quinone; BP-3,6-quinone = benzo[a]pyrene-3,6-quinone; DMBA = 7,12-dimethylbenz[a]anthracene; DMN = dimethylnitrosamine; EGTA = ethyleneglycol-bis(β-aminoethyl ether)*N,N'*-tetraacetic acid; PBS = phosphate-buffered saline.

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(Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, N.Y.), 2 m*M* L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml. All culture vessels were kept in humidified incubators at 37°C with continuous flow of 5% CO_2 in air.

Cell-mediated mutagenesis.—Bladder cells (3.5-4.0×10^6) were seeded into 35-mm petri dishes that had been seeded 18 hours earlier with 10^5 V79 cells. The bladder cells were allowed to attach overnight, the medium and any unattached cells were then removed, and fresh medium containing the chemicals was added. About 10^6 bladder cells attached to the surfaces of the V79 cells in each dish. After 48 hours, the bladder cells and V79 cells were dispersed with 0.25% trypsin–0.5 m*M* EGTA for reseeding. The V79 cells were easily distinguished from the larger bladder cells and were counted on a hemacytometer. Simultaneously, 100 V79 cells in 5 ml medium per 60-mm petri dish were seeded to determine cloning efficiency (8 dishes/point). The bladder cells did not reseed.

The procedure for determining 6-thioguanine-resistant mutants has been described (*13*). The procedure involved reseeding 5×10^5 V79 cells in 25 ml medium into a 75-cm^2 T-flask and allowing the cells to grow for 4 days. The cells were then reseeded at 2×10^4 cells in 4 ml medium into 60-mm petri dishes (20 dishes/point). Cells were again seeded for cloning efficiency as described above. Two days after plating for mutant selection, 6-thioguanine in 1 ml medium was added to each dish to give a final concentration of 5 μg/ml. This 6-day expression time allowed for an optimal number of mutants (*13*). These mutation dishes were fixed and stained 14–17 days later without interim medium change. Cloning efficiency dishes were fixed and stained 6–7 days after reseeding. The mutation frequency was calculated per 10^6 survivors based on the second cloning efficiency and the number of cells seeded for mutant selection. Intermittently, resistant colonies were isolated, grown for 30 days in the absence of 6-thioguanine, and then verified for growth in the presence of the selective agent.

BP metabolite assay.—[G-^{3}H]BP (24–40 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.) was purified by high-pressure liquid chromatography with a Zorbax-ODS column [4.6 mm (internal diameter) × 24 cm; DuPont Co., Wilmington, Del.]. Nonradioactive BP was added to adjust the specific activity.

For the metabolism studies, 3–4×10^6 bladder cells were seeded per 35-mm dish (without V79 cells) and allowed to attach overnight. The medium was then removed, and 3 ml medium containing 1 μg BP/ml (≈4 μCi) was added. After 48 hours, the medium was removed and the cells were trypsinized and combined with the medium for lysing by freeze-thawing. The combined cells and medium were extracted with ethyl acetate:acetone (2:1) and centrifuged for 10 minutes at 1,100×g. An aliquot consisting of 80% of the organic layer was removed, evaporated under nitrogen, and stored at −80°C. Samples were redissolved in 150 μl methanol:acetone (2:1) containing the appropriate unlabeled BP metabolite standards, and 10 μl of the solution was injected into a DuPont model 848 high-pressure chromatograph fitted with a 4-mm × 30-cm Bondapak C18 column (Waters Associates, Inc., Milford, Mass.). Metabolites were eluted at ambient temperature in methanol:water:ethyl ether (66.3:30.4:3.3, vol/vol) at a flow rate of 1.8 ml/minute. Metabolite identification and quantitation were accomplished by monitoring the effluent by UV spectroscopy and collecting the appropriate effluents for scintillation analysis. Retention times have been reported (*14*). The experiment was repeated with two flasks per point; controls without cells were also run.

Glucuronide and sulfate conjugates of BP were analyzed in the aqueous layer after organic extraction by the following procedure. Nitrogen gas was bubbled through the aqueous layer for 30 minutes to remove remaining organic solvents, and the sample volume was brought to 10 ml with deionized water. The pH was adjusted to 5 with 1 *N* acetic acid, and the sample was divided evenly into two tubes. Sulfatase (250 U, Type VIII; Sigma Chemical Co., St. Louis, Mo.) was added to one tube after 19.2 mg D-saccharic acid-1,4-lactone had been added to inhibit β-glucuronidase. β-Glucuronidase (10,000 Fishman U, Type B-10, in sodium acetate; Sigma Chemical Co.) was added to the second tube. The tubes were incubated 21 hours at 37°C in a shaking water bath. Ethyl acetate:acetone (2:1) was then added to each tube, and the organic-soluble material was analyzed as described above.

RESULTS

Bladder cell–V79 cell cocultivation.—Figure 1 shows bladder cells attached to the surface of a colony of V79 cells after 24 hours in culture. Most of the bladder cells remained rounded during the cocultivation period.

Rat bladder cell-mediated mutagenesis.—The data in table 1 demonstrate that primary rat bladder epithelial cells have the metabolic capability to activate DMBA, BP, and DMN into intermediates that mutate the V79 cells from 6-thioguanine sensitivity to 6-thioguanine resistance. None of the carcinogens in the absence of bladder cells caused mutation of the V79 cells. Also, the cocultivation of bladder and V79 cells did not enhance the spontaneous V79 background mutation frequency. Anthracene, a noncarcinogenic hydrocarbon, was not mutagenic with or without bladder cells.

BP metabolism by bladder cells.—Because BP was mutagenic in the bladder cell-mediated mutagenesis and the metabolism of this carcinogen has been well studied (*15*), BP metabolism was measured in bladder cell cultures (text-fig. 1). After 48 hours, bladder cells metabolized 6% of the BP (1 μg/ml) to organic-soluble metabolites and 32% of the BP to water-soluble metabolites. Bladder cells metabolized BP to eight distinct metabolites including diols, diones (quinones), and phenols (table 2). The major metabolites were BP-9,10-diol (7% of total), BP-7,8-diol (12%), BP-3,6-quinone

TABLE 1.—*Bladder cell (BC)-mediated mutagenesis of V79 cells with DMBA, BP, and DMN*[a]

Cells	Treatment (μg/ml)	Cloning efficiency, %[b]	6-Thioguanine-resistant mutants/10^6 survivors[c]
V79	—	85	≤3
V79+BC	—	83	≤3
V79	Anthracene (3)	82	≤3
V79+BC	Anthracene (3)	81	≤3
V79	DMBA (1.0)	74	≤3
V79+BC	DMBA (0.5)	60	26±8[d]
V79+BC	DMBA (1.0)	45	59±16[d]
V79	BP (1)	77	≤3
V79+BC	BP (1)	35	18±5[d]
V79	DMN (300)	74	≤3
V79+BC	DMN (100)	65	12±4[d]
V79+BC	DMN (300)	60	37±6[d]

[a] Each data point is the average of at least two expts, and each expt was run with 20 dishes/point.

[b] Cloning efficiency was determined immediately after the treatment period. To calculate mutation frequency, we determined the cloning efficiency from cells that were seeded 4 days after the expression time was initiated. The cloning efficiency is the No. of colony-forming cells divided by the No. of cells seeded (100 cells/dish) multiplied by 100%.

[c] Background mutation frequency (≤3 mutants/10^6 cells) was determined from at least 8 expts.

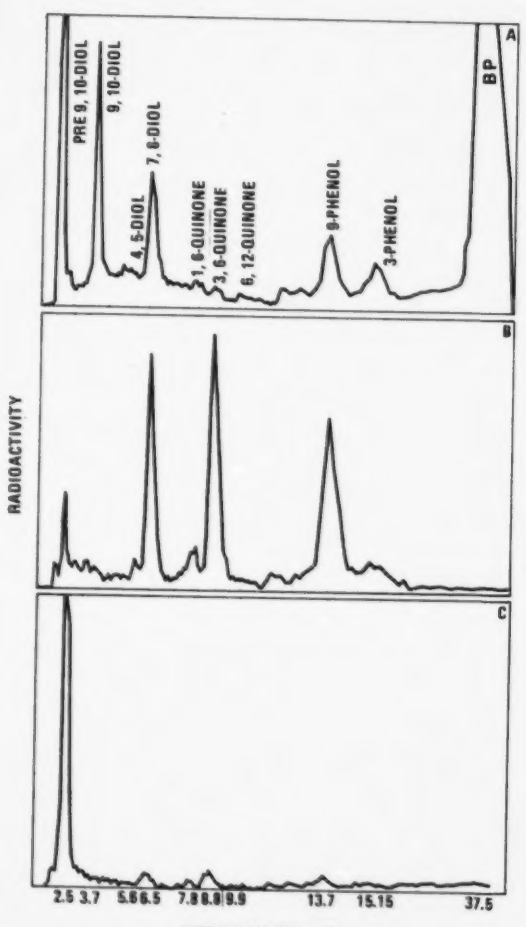
[d] Values are means ± SD.

(14%), and BP-9-phenol (7%) (text-fig. 1, table 2). BP-3-phenol, BP-1,6-quinone, and BP-4,5-diol were formed in minor amounts. Bladder cells were found to conjugate specific BP metabolites to glucuronic acid. These metabolites included BP-7,8-diol, BP-3,6-quinone, and BP-9-phenol (text-fig. 1B). Bladder cells did not conjugate any of the metabolites mentioned above to sulfate to any extent (text-fig. 1C). In all three groups of metabolites (unconjugated, glucuronic acid-conjugated, and sulfate-conjugated) formed after 48 hours of incubation, a major portion of radioactivity eluted in the void volume of the column (pre-9,10-diol fraction). This was particularly striking after sulfatase treatment; more than 85% of the radioactivity eluted in this peak (text-fig. 1C, table 2). In contrast, a similar water-soluble fraction treated with β-glucuronidase had only 10% of the β-glucuronidase-released radioactivity in the pre-9,10-diol peak (text-fig. 1B, table 2). In this chromatographic system, BP tetrols and triols elute in the pre-9,10-diol peak (data not shown). Therefore, this peak presumably represents polyhydroxylated BP metabolites arising from multiple oxidation, hydrations, reduction, and rearrangement (*15*).

DISCUSSION

In the present report, a methodology has been described for conducting cell-mediated mutagenesis studies with rat bladder epithelial cells. Studies with bovine bladder (*16*) and those reported here with rat bladder indicate that the bladder epithelium has the metabolic capability to activate chemical carcinogens. Others have also demonstrated some carcinogen-metabolizing capability in the bladder epithelium, but the reported activities were low (*17, 18*). For some bladder carcinogens, it has been proposed that metabolism and conjugations occur in the liver, and after transport in the urine, subsequent chemical or enzymatic hydrolysis in the bladder produces reactive intermediates that initiate bladder tumors (*19, 20*). The present data from in vitro studies do not refute this proposed mechanism but indicate that metabolic activation by the bladder epithelium may also play a role for certain carcinogens.

The rat bladder cells were capable of metabolizing BP to organic-soluble and water-soluble products. The presence of BP-7,8-diol in the metabolites produced by bladder cells suggests that the bladder epithelium can activate BP to 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene-



TEXT-FIGURE 1.—High-pressure liquid chromatogram of BP metabolites from bladder cells. A) Unconjugated, B) aqueous fraction after β-glucuronidase treatment, C) aqueous fraction after sulfatase treatment. Cells were treated for 48 hr with [^{3}H]BP (1 μg/ml), and the metabolites were extracted and analyzed as described in "Materials and Methods."

TABLE 2.—*BP metabolites formed by bladder cells*

Type of metabolite	BP metabolite formed, pmol[a]									
	Pre-9,10-diol	9,10-Diol	4,5-Diol	7,8-Diol	1,6-Quinone	3,6-Quinone	6,12-Quinone	9-Phenol	3-Phenol	Total
Unconjugated	639±27	292±4	49±14	212±3	393±8	26±15	10±1	74±8	44±6	1,384±73
Glucuronide-conjugated	189±3	49±3	39±5	371±15	121±23	621±46	33±11	245±39	78±17	1,746±150
Sulfate-conjugated	1,827±56	26±6	21±5	46±15	31±5	80±16	11±4	41±6	31±24	2,113±125
Total (%)	2,655 (50)[b]	367 (7)	108 (2)	629 (12)	192 (4)	726 (14)	54 (1)	360 (7)	152 (3)	5,243 (100)

[a] BP metabolite levels were determined after incubation of [^{3}H]BP (1 μg/ml) with bladder cells (6–8×10^6 cells/point) for 48 hr. *See* "Materials and Methods" for details. Values are means ± variance for duplicate samples after background subtraction.
[b] This material may represent triols and tetrols as well as a BP glucuronide initially present as a glucuronide–sulfate conjugate.

9,10-oxide, which is a presumed biologically active intermediate of BP (*15*). The bladder cells are also capable of conjugating the BP metabolites to glucuronic acid and sulfate. BP quinones, especially BP-3,6-quinone, are also formed in cultures of bladder cells. They are, however, rapidly reduced and conjugated to glucuronic acid. This has also been reported to occur in tracheal cells in culture (*21*).

The specific activity of BP metabolism in bladder cells is 904 pmol/24 hours/10^6 cells. This compares to values of 10,080 pmol/24 hours/10^6 cells for BP-transformable mouse embryo fibroblasts (data not shown) and of 2,250 pmol/24 hours/10^6 primary lung cells, which activate BP to mutagenic forms (Langenbach R, Nesnow S, Tompa A, Gingell R, Kuszynski C: Manuscript in preparation). Even though the ability of bladder cells to metabolize BP is less than that of other cell types, 629 pmol BP-7,8-diol was formed in 48 hours, the time in cocultivation with V79 cells.

We are interested in using the cell-mediated mutagenesis approach to study the organ specificity of chemical carcinogens and to compare relative mutagenic activities with cells from different organs. Table 3 shows the relative carcinogenic activities of DMN, DMBA, and BP in the liver, lung, and bladder (*22–25*) and the relative mutagenic activities of the chemicals

TABLE 3.—*Relative carcinogenic and cell-mediated mutagenic activities of DMN, DMBA, and BP*[a]

Chemical	Liver		Lung		Bladder	
	Carcinogenic activity	Mutagenic activity	Carcinogenic activity	Mutagenic activity	Carcinogenic activity	Mutagenic activity
DMN	S	++	W	–	?[b]	+
DMBA	W	+	S	++	W	+
BP	W	–	S	++	?	+

[a] The relative carcinogenic activity was assigned from (*22–25*): W=weak, S=strong. The mutagenesis data are from [(*1–4*); Langenbach R, Nesnow S, Tompa A, Gingell R, Kuszynski C: Manuscript in preparation]: DMN (100 μg/ml), DMBA (1 μg/ml), and BP (1 μg/ml). Relative mutagenic activity: –=<4 times greater than background, +=4–20 times background, ++=>20 times background.
[b] Epidemiology data suggest DMN to have a role in human bladder cancer (*28*).

in the liver, lung, and bladder cell-mediated systems (*1–4*). For liver and lung, there is general agreement between the target organ for carcinogenesis and mutagenic activity in the cell-mediated system with all three carcinogens. In the bladder cell-mediated system, all three carcinogens were mutagenic, with activities in between target and nontarget organ responses. However, rodent bladder carcinogenesis data for these carcinogens are limited. DMBA has been reported to cause focal hyperplasia and papillomas in the bladder (*22*) and to transform bladder epithelial cells in organ culture (*26*). The finding that bladder epithelial cells can activate DMN is of importance because of the recent report of Radomski et al. (*27*) on its presence in the urine of humans with certain types of urinary tract infections. While DMN has not been shown to induce bladder cancer in animals or humans, people with frequent urinary tract infections have an increased incidence of bladder cancer (*28*). BP has not been shown to induce bladder cancer, although correlations between bladder cancer and smoking have been shown (*29*), and BP along with other known carcinogens is a component of cigarette smoke. The possible involvement of BP glucuronides in bladder cancer initiation has been suggested (*30*). The present studies do demonstrate that bladder epithelium can activate these chemical carcinogens, and studies are under way to investigate some of the known, more specific bladder carcinogens.

In summary, we have demonstrated in the present report that 1) rat bladder epithelial cells are capable of metabolizing at least some chemical carcinogens and 2) the cell-mediated mutagenesis approach can be used to study organotropic effects of certain chemical carcinogens under controlled conditions. We are currently utilizing this approach to investigate biologic processes that may be involved in determining organ specificity.

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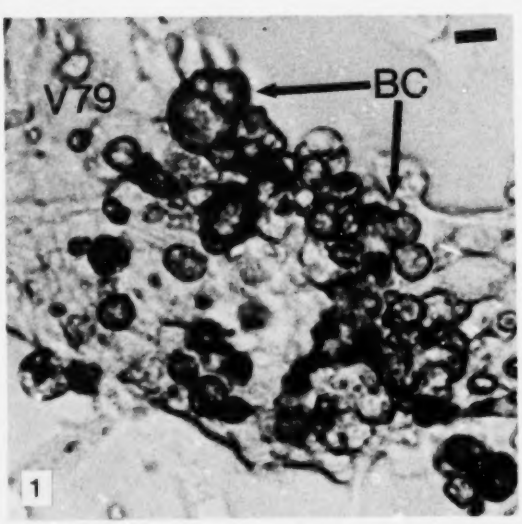

Figure 1.—Bladder cells (BC) on a colony of V79 cells (V79). Phase contrast. *Bar* = 10 μm. × 650